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CLINICAL AND PATHOGENETIC FEATURES OF *ETV6*-RELATED THROMBOCYTOPENIA WITH PREDISPOSITION TO ACUTE LYMPHOBLASTIC LEUKEMIA

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Short title: *ETV6*-related thrombocytopenia

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ABSTRACT

ETV6-related thrombocytopenia (*ETV6*-RT) is an autosomal dominant thrombocytopenia that has been recently identified in a few families and has been suspected to predispose to hematological malignancies. To gain further information on this disorder, we searched for *ETV6* mutations in the 130 families with inherited thrombocytopenia of unknown origin from our cohort of 274 consecutive pedigrees with familial thrombocytopenia. We identified 20 *ETV6*-RT patients from 7 pedigrees. They have 5 different ETV6 variants, including three novel mutations affecting the highly conserved E26 transformation-specific domain. The relative frequency of *ETV6*-RT resulted 2.6% in the whole case series and 4.6% among the families with known forms of inherited thrombocytopenia. The degree of thrombocytopenia and bleeding tendency of *ETV6*-RT patients were mild, but 4 subjects developed B-cell acute lymphoblastic leukemia during childhood, resulting in a significantly increased incidence compared to the general population. Clinical and laboratory findings did not identify any peculiar defects that can be used to suspect this disorder by routine diagnostic workup. However, at variance with most inherited thrombocytopenias, platelet size was not enlarged. *In vitro* studies revealed that patients' megakaryocytes have defective maturation and impaired proplatelet formation. Moreover, *ETV6*-RT platelets have reduced ability to spread on fibrinogen. Since also the dominant thrombocytopenias due to mutations in *RUNX1* and *ANKRD26* are characterized by normal platelet size and predispose to hematological malignancies, we suggest that mutation screening of *ETV6*, *RUNX1* and *ANKRD26* should be performed in all the subjects with autosomal dominant thrombocytopenia and normal platelet size.

INTRODUCTION

Until the end of the last century, only a few forms of inherited thrombocytopenia (IT) were known, all of them extremely rare and characterized by severe bleeding tendency. Since then, knowledge of ITs has greatly improved and we presently know at least 26 disorders caused by mutations in 30 genes.[1,2] This advancement of knowledge revealed that most patients with ITs have only mild or moderate thrombocytopenia, with trivial bleeding episodes or no bleeding at all. However, it also became apparent than many patients are exposed to the threat of acquiring additional defects that worsen their quality of life or can even be fatal. Subjects with *MYH9*-related disease are predisposed to proteinuric nephropathy evolving into end stage renal failure, those with congenital amegakaryocytic thrombocytopenia always develop bone marrow aplasia, while patients with *ANKRD26*-related thrombocytopenia (*ANKRD26*-RT) or familial platelet disorder with predisposition to acute myeloid leukemia (AML) due to *RUNX1* mutations (FPD/AML) have increased risk of AML and myelodysplastic syndromes (MDS). Thus, bleeding is no longer the unique problem of IT patients.

In 2015, four independent studies showed that mutations in the gene *ETV6* are responsible for a new form of IT and suggested that *ETV6*-related thrombocytopenia (*ETV6*-RT) predisposes to acute lymphoblastic leukemia (ALL)[3-6]. However, only a few families have been reported so far and the clinical and laboratory features of *ETV6*-RT remain poorly defined.

In order to gain further information on this disorder, we screened for *ETV6* mutations 130 consecutive unrelated propositi with IT of unknown origin and identified 7 affected families. Two of these pedigrees have been briefly reported in a previous paper.[4] Here we describe the features of 20 affected subjects, who form the largest cohort of *ETV6*-RT patients collected so far. As these patients were identified by screening a wide series of consecutive, unselected probands with familial thrombocytopenia, we could estimate the relative frequency of *ETV6*-RT among ITs and the risk of hematological malignancies associated with this condition. By reporting in details the clinical and laboratory features of these patients, we provide the tools to suspect this disorder in the routine diagnostic workup of probands with ITs. Finally, we discuss the pathogenesis of *ETV6*-RT by investigating for the first time megakaryocytes (Mks) differentiated from hematopoietic progenitors of patients, and by the functional characterization of *ETV6*-RT platelets.

METHODS

Patients

From 2003 to 2014, we analyzed at the IRCCS Policlinico San Matteo Foundation of Pavia (Italy) 274 consecutive unrelated probands with familial thrombocytopenia. By the application of a well-defined diagnostic algorithm for ITs,[1] we made a molecular diagnosis in 144 of these families, whereas 130 probands remained without a definite diagnosis as they did not fit the criteria for any known IT. These 130 consecutive propositi with IT of unknown origin have been screened for mutations in *ETV6*. Whenever *ETV6* mutations were identified, the available relatives of probands have been investigated.

Bleeding tendency was measured according to the International Society on Thrombosis and Haemostasis (ISTH) bleeding assessment tool (BAT) score.[7]

The institutional review board of the San Matteo Foundation approved the study and all subjects or their legal guardians signed written informed consent in accordance with the Declaration of Helsinki.

Mutation screening and RT-PCR

Genomic DNA and RNA were extracted from peripheral blood. The *ETV6* gene was analyzed using Sanger and whole exome sequencing. Methods of mutation screening and RT-PCR are detailed in the supplementary information.

Bioinformatic tools and analysis of the structures

The bioinformatic tools used to evaluate missense variants and methods of analysis of ETV6 structure are reported in supplementary information.

Basic blood cell studies

Blood cell counts were evaluated by electronic counters. Parameters relative to platelet diameter were measured by software-assisted image analysis on blood smears as reported.[8] The following previously defined parameters were computed: mean platelet diameter (MPD), platelet diameter distribution width (PDDW), platelet diameter large cell ratio (PDLCR), and platelet diameter small cell ratio (PDSCR).[8] The percentage of large platelets has been also estimated empirically, as previously reported[8] and detailed in supplementary information. Surface expression of platelet glycoproteins (GPs) was investigated by flow cytometry as reported, whereas platelet aggregation was evaluated by the densitometric method of Born.[9] The used antibodies and platelet agonists are listed in supplementary information.

Platelet activation

Platelet activation in response to ADP or TRAP was investigated by flow cytometry as reported.[10] The protocol is described in details in supplementary information.

Platelet adhesion and spreading

Platelet adhesion and spreading on the subendothelium components of the extracellular matrix type I collagen, von Willebrand Factor, or fibrinogen, were investigated as previously described[11,12] and as detailed in supplementary information.

Investigation of megakaryocytes

Mks were differentiated in vitro from peripheral blood $CD45^+$ cells as previously reported.[13,14] Morphological analysis of Mks was performed by phase-contrast and fluorescence microscopy, while the percentage of fully differentiated Mks and Mk ploidy at the end of the culture were investigated by flow cytometry.[14,15] Proplatelet yields were evaluated both in suspension and in adhesion on fibrinogen at the end of the culture, as previously described.[13,16] Methods are reported in supplementary information.

Statistical analysis

Data are presented as mean and SD or range. Statistical comparisons were performed by two-tailed Student t test. Incidences of hematologic malignancies (per 100 000 person years) together with their exact 95% confidence intervals (95%CI) were computed.

RESULTS

Mutation screening

The analysis of the *ETV6* gene allowed us to identify 5 different heterozygous variants in 7 unrelated pedigrees. Two variants (c.641C>T/p.P214L and c.1252A>G/ p.R418G+p.N385Vfs*7) were previously reported in two families (Figure 1A, families B and G).[4] The remaining three novel variants are two missense alterations and one deletion.

The two missense variants, c.1105C>T (p.R369W) and c.1138T>A (p.W380R) segregate in the affected family members and are not present in healthy relatives (Figure 1A). They are absent in public genomic databases, such as dbSNP (www.ncbi.nlm.nih.gov/SNP), 1000 genomes (www.1000genomes.org), and Exome Aggregation Consortium (www.exac.broadinstitute.org). Multiple-sequence alignment indicated that they affect highly conserved amino acid residues (data not shown). They are predicted to be deleterious for protein function according to different tools (Supplementary Table S1). Moreover, the CADD scores were 26.1 and 23.1 for p.R369W and p.W380R, respectively. Both mutations map in the E26 transformation-specific (ETS) domain which is in the C-terminal half of the protein (residues 337-421). Analysis of the coordinates of the ETS domain of ETV6 (2DAO) (numbered in pdb C8 and L112, so that R369 and W380 correspond to R39 and W50) shows that W380 is well buried in the hydrophobic core and surrounded by a number of hydrophobic residues, such as L341 and M394 (L11 and M64 in the structure) (Figure 1E). W380 is also close to the side chains of H383 and K384 (H53 and K54). Its substitution to an arginine will greatly destabilize the structure by creating both an uncompensated cavity in the hydrophobic core and an electrostatic repulsion with nearby positively charged residues. Residue R369 is well exposed on the protein surface and is predicted to form an electrostatic interaction with the spatially nearby E361 (E31). Its substitution with a tryptophan could destabilize the fold by abolishing this interaction. Alternatively, this residue could be implicated in protein-protein interactions. In this case, its substitution with a much bulkier and uncharged residue could be deleterious.

The c.1153-1_1165del deletion variant removes the last "G" nucleotide of intron 6 and the first 13 nucleotides of exon 7. To provide the effect of this deletion, we carried out RT-PCR on the three affected individuals of family F. Sequencing analysis of the altered 721 bp product showed skipping of exon 7 (r.1153_1253del/p.N385Vfs*7; Figure 1B) resulting in truncation of the ETS domain. Since the 721 bp band is fainter than the wild type product (822 bp), we cannot exclude that the alternatively spliced mRNA is partially degraded. Inspection of the intron 6/exon 7 genomic boundary reveals repeats that are likely to be involved in non-allelic homologous recombination leading to micro deletions/duplications (Figure 1C).

The 7 families reported in figure 1A formed our cohort of 20 affected individuals who have been studied for the phenotypic characterization of *ETV6*-RT.

Clinical picture

A mild bleeding tendency was present in 12 patients, whereas 8 subjects had no significant bleeding diathesis (Table 1). More common bleeding symptoms were petechiae, ecchymoses, gum bleeding, epistaxis, and menorrhagia. Thrombocytopenia was discovered in the adulthood in 5 patients, whereas it

was identified at birth in 6 patients because of the family history of low platelet count. One patient (E/I-3) was initially misdiagnosed with immune thrombocytopenia, and received steroids and underwent splenectomy at age 9 years without increase of his platelet count. Ten patients underwent 17 surgeries and 6 had tooth extractions without excessive bleeding. Four women gave birth to 6 children, three vaginally and three by cesarean section. Prophylactic platelet transfusion was deemed necessary to cover one vaginal delivery. Excessive bleeding (800 mL) was reported in another woman (patient F/I-2) who had given birth vaginally. We have no information on her platelet count and function at the time of the delivery; however, it is interesting to note that she presented defective platelet response to low doses of collagen and ADP when she was investigated at our institution (see below).

Unilateral polydactyly was observed in one patient, mitral valve prolapse in two subjects, and renal ectopia in one. So, no recurrent extra-hematological abnormalities have been identified.

Four patients from 4 families developed B-cell ALL (common ALL in three cases, not better defined in one) during childhood. Conventional cytogenetic analysis resulted normal in three cases, while patient E/II-1 had hyperdiploid ALL; the search for the *ETV6-AML1* transcript was performed in one patient (E/II-1) with normal findings. The incidence of ALL in our case series was 731.3 per 100 000 (95%CI, 274.5-1948.4), while it is 1.4 per 100 000 in the general population according to the National Cancer Institute.[17] Three patients obtained remission after conventional chemotherapy, and one after hematopoietic stem cell transplantation (HSCT) from unrelated donor. The patient E/I-3, who had a history of isolated thrombocytopenia since childhood (Table 1), at age 37 developed increased hemoglobin level (19.0 gr/dL, hematocrit 56%), with mild leukocytosis and thrombocytosis. The *JAK2*V617F mutation was identified and a diagnosis of polycythemia vera was made.

A history of non-hematological neoplasms was present in three patients. The patient B/II-1 had breast fibroadenoma at age 35 and meningioma at age 42. The patient G/I-2 had breast carcinoma at age 49, while the patient F/II-2 had breast fibroadenoma at age 14.

Blood cell counts and peripheral blood film examination

Table 1 reports the blood cell counts obtained at the last examination for 18 patients, and at the last available examination before the development of polycythemia vera and before HSCT for the patients E/I-3 and E/II-1, respectively. Eleven patients had less than 100 x 10^9 platelets/L and only one less than 50 x 10^9/L. For most patients we could obtain the platelet counts measured at different ages prior to the evaluation for this study (Supplementary Table S2). Patients presented some fluctuations in their platelet

counts over time, however, none of them showed a definite trend toward improvement or worsening of thrombocytopenia during life.

Mean platelet volume (MPV) was slightly reduced in 4 cases and normal in the other 14 evaluable patients (Table 1). Peripheral blood film examination in 16 patients showed that MPD was similar to that of healthy subjects, confirming that average platelet size is consistently normal in *ETV6*-RT patients (Table 2). We found very mild but significant increases of PDDW and PDLCR, which indicate that a mild platelet anisocytosis and a slightly increased proportion of large platelets were frequent features of the investigated patients. In agreement with previous findings,[8] the empirical measurement of the percentage of platelets larger than half an erythrocyte gave similar results to the assessment of PDLCR by image analysis (data not shown). Conversely, the increased mean PDDW detected by image analysis did not correspond to increased mean platelet distribution width (PDW) values obtained by automated cell counts (not shown).

Mild anemia has been observed in one patient with iron deficiency (A/II-1). Mean corpuscular volume (MCV) was reduced in this subject, increased without any apparent cause in five subjects, and within the normal range in the remaining patients. White blood cell count was normal in all the cases.

***In vitro* platelet studies**

Platelet aggregation. Among the 11 investigated patients, the three patients from family F had mildly reduced platelet aggregation after stimulation with collagen 4 µg/mL and ADP 5 µM, while individual C/II-1 showed a slightly reduced response to ristocetin 1.5 mg/mL (Table 3). However, all patients had completely normal responses to higher concentrations of these agonists (collagen 20 µg/mL, ADP 20 µM, ristocetin 3 mg/mL, data not shown), indicating that, if present, the aggregation defects were mild.

Platelet flow cytometry. As shown in Table 3, flow cytometry performed in 11 patients did not identify any consistent defect of the major GPs of the platelet surface.

Platelet activation. Overall, the surface expression of activated GPIIb-IIIa and P-selectin and the reduction of GPIbα upon stimulation of platelets with ADP or TRAP, were not significantly different in 11 *ETV6*-RT patients with respect to controls (Supplementary Figure 1). A mild reduction of activated GPIIb-IIIa expression (52 to 65% of controls) after stimulation with TRAP was observed in three patients.

Platelet adhesion and spreading. *In vitro* adhesion of platelets from 7 patients to subendothelium components of the extracellular matrix was not different from that of controls. However, the ability of

ETV6-RT platelets to spread on fibrinogen was consistently and significantly reduced, while spreading on collagen and von Willebrand Factor was normal (Table 4).

In vitro culture of megakaryocytes and assessment of proplatelet formation

In vitro culture of Mks was performed in 8 patients and 8 healthy subjects. After 14 days of culture, expression levels of the major Mk differentiation surface markers (GPIIIa, GPIIb and GPIbα) were similar to those of healthy controls (Figures 2A and 2B). Conversely, Mk ploidy was significantly lower in patients than controls (Figure 2C), and this was paralleled by differences in Mk diameters (Figure 2D). The analysis of proplatelets formation revealed that Mks from patients elongated proplatelet shafts of shorter length and with decreased number of branches compared to controls. Further, the percentage of proplatelet-forming Mks was significantly reduced. In contrast, the size of proplatelet tips was similar to that of controls (data not shown). Similar results were obtained with Mks in suspension (Figures 3A and 3B) and in adhesion on fibrinogen (Figures 3C and 3D).

DISCUSSION

This study reports the molecular and phenotypic characterization of 7 families with germline mutations in *ETV6*. In addition to the variants previously reported,[4] we identified three novel alterations, which are likely to be pathogenetic. The two novel missense variants (p.R369W and p.W380R) segregated within the families, are absent in public genomic databases, and are expected to be deleterious for protein function by bioinformatic tools and analysis of protein conformational structure. ETV6 is a modular protein which contains a PNT and an ETS domain sandwiched between regions of potential intrinsically unstructured nature. Both p.R369W and p.W380R affect the ETS domain, a conserved region that directly interacts with DNA consensus sequences. We have shown that the role of W380 is structural, being surrounded by hydrophobic residues in the domain hydrophobic core. Its substitution to an arginine will thus greatly destabilize the domain structure. Residue R369 is involved in an electrostatic interaction and possibly in protein-protein interactions.

It is important to note that the somatic p.R369W has been previously associated with chronic myelomonocytic leukemia (CMML), colorectal cancer, and childhood leukemia.[5,6] Moreover, Zhang et al. previously reported one *ETV6*-RT pedigree carrying a different germline missense variant affecting the same residue (p.R369Q). Similarly to our patients with the p.R369W, the subjects with p.R369Q presented mild thrombocytopenia and normal platelet morphology.[6] Among the 8 members of the p.R369Q pedigree, one CMML at age 82 and one colorectal cancer at age 43 were reported,[6] whereas we did not observe neoplasms in our p.R369W patients at a median age at evaluation of 30 years. Finally, both p.R369Q and

p.R369W have been associated with genetic predisposition to childhood ALL.[3] These observations suggest that arginine 369 is a mutational hot spot.

Regarding the c.1153-1_1165del variant, RT-PCR demonstrated that it affects the splicing process leading to skipping of exon 7 (p.N385Vfs*7). The same alternative splicing was also caused by the c.1153-5_1153-1del mutation,[5] which, as c.1153-1_1165del, is likely to derive form non-allelic homologous recombination between repetitive sequences present at the intron 6/exon 7 boundary. The skipping of exon 7 is also determined by the c.1252A>G substitution.[4] Affecting the second to last nucleotide of exon 7, this allele is associated with both correctly (p.R418G) and alternatively (p.N385Vfs*7) spliced mRNA.[4]

Of the 10 different mutant forms identified so far in *ETV6*-RT families, the p.P214L is the only one which does not affect the ETS domain (Figure 1D), but instead alters a less conserved central domain that interacts with several transcription repressors further controlling expression of the target genes. Contrary to the other germline mutations, which are mainly private, this substitution was responsible for *ETV6*-RT in 4 of the 14 families characterized so far, indicating that it represents another potential mutational hot spot.

ETV6 is a transcriptional repressor involved in the embryonic development and hematopoietic regulation.[18] In particular, animal studies suggested that *ETV6* has two independent roles in mouse hematopoiesis: on the one hand it is required for survival of hematopoietic stem cells, on the other it promotes the late phases of megakaryopoiesis. Interest in *ETV6* greatly increased at the end of the last century after demonstration that its deregulation due to rearrangement, fusion or deletion is involved in hematologic malignancies.[19,20] Moreover, somatic mutations in *ETV6* were recently found in a variety of hematologic neoplasm, including AML, T and B cell ALL, mixed-phenotype acute leukemia, MDS, chronic lymphocytic leukemia and chronic myelogenous leukemia.[21] Even more recently, targeted sequencing of *ETV6* in 4405 childhood ALL cases identified 31 germline variants potentially related to leukemia in 35 cases.[3] Based on this evidence, it is not surprising that the 4 studies that identified *ETV6*-RT in 41 subjects from 9 families found that 16 patients (39%) had hematological malignancies, with 12 patients (29%) developing ALL.[3-6] Of note, 11 of 12 subjects with ALL were children. The other observed blood neoplasms in *ETV6*-RT patients were mixed-phenotype acute leukemia, multiple myeloma, MDS and CMML.

We found that 4 of 20 consecutive patients with *ETV6*-RT (20%) developed ALL during childhood, thus confirming that early leukemic transformation is a major risk of these patients. Moreover, we observed that one patient developed *JAK2* positive polycythemia vera at age 37, supporting the previous hypothesis that *ETV6*-RT predisposes not only to ALL, but also to other blood neoplasms. The frequency of hematologic malignancies is lower in our study than in the previous ones (25% versus 39%). This is explained by the fact that, in the previous investigations,[3-6] the occurrence of hematologic malignancies was one of the criteria for the recruitment of patients, while we examined a series of consecutive, unselected patients with IT of

unknown origin. This approach appears more suitable to provide a reliable estimation of the incidence of hematologic neoplasms among *ETV6*-RT patients. Of course, the analysis of a larger series of patients is needed to confirm this figure.

Similarly to this study, we previously searched for *ANKRD26* mutations a large series of unselected patients and revealed that 10 of 118 (8%) subjects with *ANKRD26*-RT had developed myeloid malignancies.[22] Thus, hematological malignancies seem much more frequent in *ETV6*-RT than *ANKRD26*-RT. The risk of malignancies appears even higher in FPD/AML, since over 40% of patients had myeloid neoplasms.[23] However, as discussed for *ETV6*-RT, also *RUNX1* mutational screening was generally performed in pedigrees with hematological malignancies,[24] and it is therefore likely that the incidence of transformation has been overestimated. However, each patient with an IT caused by mutations in *ETV6*, *RUNX1* or *ANKRD26* has a relevant risk of hematological malignancies, and recognizing these patients is required not only to provide effective genetic counseling and appropriate follow-up, but also to give an appropriate treatment to patients who developed blood neoplasms and need HSCT. In fact, as shown in different disorders predisposing to myeloid malignancies,[25] the use of an affected family member as the donor would entail the risk of developing malignancies once again.

ETV6-RT is a relatively frequent form of IT. In fact, in our series of 274 consecutive propositi, *ETV6*-RT was identified in 7 families and had, therefore, a relative prevalence of 2.6% in the whole case series, and of 4.6% in the series of probands with known ITs (7/151). In our cohort, the frequency of *ETV6*-RT was lower only to that of monoallelic Bernard-Soulier syndrome (BSS, 12.2% in the whole series), *MYH9*-related disease (11.4%), *ANKRD26*-RT (9.4%), and biallelic BSS (5.7%). Since most of our patients with monoallelic BSS had the Ala156Val mutation of GPIbα (Bolzano mutation), which is exclusive of the Italian population,[26] it is expected that the relative frequency of *ETV6*-RT is even higher in other countries.

Our study did not identify any peculiar feature that can be used to raise the suspicion of *ETV6*-RT by routine diagnostic workup and the diagnosis remains therefore difficult. In particular, a previous investigation, which reported also 5 patients who have been re-evaluated for this study, suggested that red blood cell (RBC) macrocytosis is a feature of the *ETV6*-RT phenotype.[4] In that investigation, the percentage of patients with increased MCV was 40%,[4] whereas it resulted 25% in the present study. Concerning the 5 patients reported in both studies, RBC macrocytosis had been found in two individuals by the previous examination but was not confirmed by the present evaluation. Of note, the absolute MCV values were similar in the two studies (mean 94.5 fL with SD 3.8 vs. mean 93.3 fL with SD 8.9) and the discrepancy in the percentage of patients with RBC macrocytosis resulted from the different upper limits of normal range used in the two investigation (95 fL in the previous study and 98 fL in the present one, according to the normal ranges of

the different laboratories). On the whole, these findings indicate that RBC macrocytosis is present in a minority of patients with *ETV6*-RT, and suggest that it may be inconstantly found in the same patients over time. Thus, RBC macrocytosis seems to have a limited diagnostic value to recognize this condition. Moreover, we did not identify any distinguishing defect of major platelet GPs or *in vitro* platelet aggregation. Also evaluation of peripheral blood films did not reveal any morphological abnormality, except for mild platelet anisocytosis. However, at variance with most ITs, MPD and MPV were consistently normal in *ETV6*-RT, and it is just the normal size of platelets that should raise suspicion of this condition in subjects with an autosomal dominant thrombocytopenia. In fact, the other dominant ITs with this feature are FDP/AML, *ANKRD26*-RT, and *CYCS*-RT. Of note, *CYCS*-RT is a very rare condition described so far in only two pedigrees,[1] whereas the other two disorders are more frequent and, as *ETV6*-RT, predispose to hematologic malignancies. Thus, we suggest that all subjects with a dominant IT and normal platelet size should be tested for mutations in *ETV6*, *RUNX1*, and *ANKRD26*, in order to recognize one of these predisposition syndromes.

The psychological impact of receiving a diagnosis of *ETV6*-RT, as well as of FPD-AML or *ANKRD26*-RT, should be carefully considered by physicians. We suggest that each patient, before undergoing diagnostic workup for thrombocytopenia of suspected genetic origin, is correctly informed about the possibility to receive a diagnosis that implicates the risk of malignancies, and has the chance to state in advance if he wants to receive information about the risk of neoplasms, for himself as well as for his progeny.

Our study investigated for the first time *in vitro* megakaryopoiesis of *ETV6*-RT patients. We showed that *ETV6* pathogenetic variants impair Mk maturation, as demonstrated by the production of smaller Mks with decreased ploidy. These immature Mks showed an impaired ability to extend fully developed proplatelets, providing an explanation for thrombocytopenia. These findings seem consistent with the results of the studies in mice, which suggested a role for *ETV6* in terminal Mk maturation,[18] and with the findings obtained with Mk differentiated from human $CD34^+$ cells transduced with some *ETV6* variants.[4] We also had the possibility to study in detail the function of platelets in a substantial number of patients. Although we did not identify any consistent defect of *in vitro* platelet aggregation, activation and adhesion, we found that the ability of platelets to spread on fibrinogen was reduced in all the investigated patients. As the platelet expression of GPIIb-IIIa was normal, this finding suggests that mutations in the ETV6 transcription factor alter the expression of one or more proteins involved in the GPIIb-IIIa-mediated platelet outside-in signalling after interaction with fibrinogen. Moreover, this defect could contribute to the bleeding diathesis observed in some *ETV6*-RT individuals. In fact, although the degree of bleeding was always mild, the proportion of patients with spontaneous bleeding (60%) appeared globally high with respect to the very mild degree of thrombocytopenia.

In conclusion, our study showed that monoallelic *ETV6* mutations cause a relatively frequent form of IT and confirmed that affected subjects have mild bleeding tendency but propensity to hematological malignancies, in particular ALL. Since *ETV6*-RT is one of the few autosomal dominant forms of IT without platelet macrocytosis, the screening for *ETV6* mutations is recommended in all patients with these characteristics.

AUTHORSHIP CONTRIBUTIONS

MS, CLB, AP, and AS designed research, performed research, interpreted data, and wrote the manuscript; FM, PN, AB and CA preformed research, interpreted data and wrote the manuscript; FP, DDR, CM, CG, TP, VB, MF, SB, MD, CDB, KSK, LR, GL, and SP performed research and interpreted data; all the authors critically revised the manuscript and accepted the final version.

DISCLOSURE

The authors declare no conflicts of interest.

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Table 1. Main characteristics of the investigated patients.

Family/ Individual	ETV6 mutation[2]	Age[3], y/ Gender	Age at diagnosis[4], y	ISTH BAT score[5]	Platelets, x10^9/L	MPV, fL[6]	MPD, µm[7]	Hgb, g/dL	MCV, fL[8]	WBC, x10^9/L	Neutrophils x10^9/L	Hematological malignancies
A/I-1	c.641C>T p.Pro214Leu	57/M	30	3	115	8.8	2.44	14.6	99	7.13	4.9	
A/II-1		20/F	birth	7	59	8.6	2.24	10.4	68	4.98	2.3	
A/II-2		27/F	birth	3	82	8.2	2.23	13.6	98	5.5	3.39	Common ALL at age 7
B/I-2[1]		43/F	14	0	115	10	2.82	11.1	88	5.02	1.75	B-cell ALL at age 15
B/II-1[1]		15/M	birth	3	66	10.4	2.89	14.0	91	5.36	1.18	
B/II-2[1]		18/F	2	0	44	10.1	3.26	13.1	97	4.04	1.42	
C/I-1	**c.1105C>T p.Arg369Trp**	48/M	38	3	112	na	2.73	15.4	103	6.3	4	
C/II-1		13/M	3	0	87	na	2.53	14.1	86	3.84	1.81	
D/I-1		53/M	47	0	110	8.4	2.42	13.7	97	5.4	2.84	
D/II-1		7/F	1	0	109	9.2	2.28	12.6	79	6.82	1.87	
E/I-1	**c.1138T>A p.Trp380Arg**	37/F	8	0	105	8.1	na	14.2	97	7.50	5.2	
E/I-3		42/M	5	4	55[9]	9.1[9]	na	14.9[9]	94[9]	8.0[9]	6.1[9]	*JAK2*V617F+ PV at age 37
E/I-4		45/M	20	0	93	7.9	na	16.9	101	8.30	4.24	
E/II-1		20/M	4	2	60[10]	8.0[10]	2.73[10]	14.8[10]	86[10]	5.11[10]	1.97[10]	Common ALL at age 7
E/II-3		13/M	birth	4	99	7.4	na	14.0	90	6.15	2.45	
F/I-2	**c.1153-1_1165del r.1153_1253del p.Asn385Valfs*7**	49/F	7	2	105	8.9	2.55	13.4	107	7.11	4.4	
F/II-1		12/F	birth	1	57	8.6	2.40	14.2	97	6.59	4	
F/II-2		17/F	birth	2	70	8.7	2.36	14.4	97	8.24	5.3	Common ALL at age 3
G/I-2[1]	c.1252A>G p.Arg418Gly + p.Asn385Valfs*7	51/F	20	0	101	7.6	3.17	13.6	97	4.71	2.02	
G/II-1[1]		28/M	3	2	101	7.8	2.99	15.9	97	5.3	2.39	

Notes: [1] Previously reported patients. [2] Nucleotide A of the ATG translation initiation start site of the ETV6 cDNA in GenBank sequence NM_001987.4 is indicated as nucleotide +1. Novel germline mutations are in bold. [3] Age at the last evaluation: the blood parameters and bleeding score reported here have been measured at the last evaluation, unless otherwise specified. [4] Age at diagnosis of thrombocytopenia. [5] International Society on Thrombosis and Haemostasis (ISTH) bleeding assessment tool (BAT) score was calculated as previously reported (reference 7). [6] Normal range: 8-13.4 fL. [7] Normal range: 1.9-3.4

μm. [8]Normal range: 82-98 fL. [9]Parameters measured at the last available examination before the development of PV (age 35). [10]Parameters measured before chemotherapy and HSCT for the development of ALL. **Abbreviations:** na: not available; ALL: acute lymphoblastic leukemia; PV: polycythemia vera.

Table 2. Parameters of platelet diameters measured on peripheral blood films in investigated patients.

	No.[1]	**MPD, µm** mean (SD)	**PDDW, µm** mean (SD)	**PDLCR, %** mean (SD)	**PDSCR, %** mean (SD)
Family A	3	2.30 (0.12)	2.57 (0.06)	8.27 (1.77)	5.87 (3.19)
Family B	3	2.99 (0.24)	2.97 (0.21)	12.3 (4.75)	1 (0.86)
Family C	2	2.63 (0.31)	2.75 (0.49)	7 (4.24)	1.25 (1.06)
Family D	2	2.35 (0.10)	2.10 (0.14)	4.5 (4.95)	5.5 (3.53)
Family E	1	2.73	2.9	8.5	1
Family F	3	2.44 (0.10)	2.57 (0.35)	8.83 (4.07)	3.16 (1.25)
Family G	2	3.08 (0.13)	3.15 (0.49)	13 (7.07)	0.5 (0.71)
Total *ETV6*-RT patients	16	2.63 (0.17)	2.70 (0.29)*	9.23 (4.47)*	2.85 (1.77)
Healthy subjects[2]	55	2.49 (0.32)	2.18 (0.58)	3.64 (4.93)	4.35 (5.9)

Notes: [1] Number of investigated subjects. [2] Values of healthy subjects previously measured in a cohort of 55 healthy volunteers (reference 8). *p <0.01 with respect to healthy subjects.
Abbreviations: MPD, mean platelet diameter. PDDW, platelet diameter distribution width = difference from the 2.5th to the 97.5th percentile of platelet diameter distribution. PDLCR, platelet diameter large cell ratio = proportion of platelets larger than the 97.5th percentile of MPD of healthy subjects (3.9 µm). PDSCR, platelet diameter small cell ratio = proportion of platelets smaller than the 2.5th percentile of the MPD of healthy subjects (1.6 µm) (reference 8).

Table 3. In vitro platelet aggregation and surface expression of major platelet glycoproteins in investigated patients.

	Platelet aggregation, **maximal extent, % [1] -** mean (range)				**Surface expression of platelet glycoproteins, % of controls -** mean (range)			
Family	No. of investigated subjects	Collagen, 4 mcg/mL	ADP, 5 μM	Ristocetin, 1.5 mg/mL	No. of investigated subjects	GPIbα (SZ2)	GPIX (SZ1)	GPIIb (P2)
A	2	80 (71-89)	75 (66-84)	88 (76-100)	2	131 (130-132)	130 (116-144)	91 (85-97)
B	2	80 (74-87)	81 (77-85)	80 (68-93)	3	98.7 (98-99)	99 (98-100)	98.7 (97-100)
C	2	71 (69-73)	57 (44-70)	67 (57-77)	2	147.5 (143-152)	126 (122-130)	108 (94-122)
F	3	54 (50-56)	37 (35-39)	100 (100-100)	2	117.5 (110-125)	125 (121-129)	127.5 (118-137)
G	2	78 (67-90)	82 (78-87)	88 (77-100)	2	159.5 (136-165)	101 (89-113)	85 (78-92)

Note: [1] Normal ranges: collagen 66-88; ADP 43-76; ristocetin 67-90.

Table 4. In vitro platelet interaction with subendothelium molecules in 7 *ETV6*-RT patients.

	Platelet adhesion and spreading , % of controls - mean (SD)		
	No. of adhering platelets	% of spread platelets	Surface area covered by platelets
Fibrinogen	102.9 (27.6)	51.5 (33.5)*	61 (28.5)*
Collagen	107.2 (45.1)	103.6 (41.7)	122.4 (29.3)
von Willebrand Factor	80.4 (36.5)	103.8 (41.5)	120.7 (22.7)

Note: * $p < 0.01$ with respect to controls

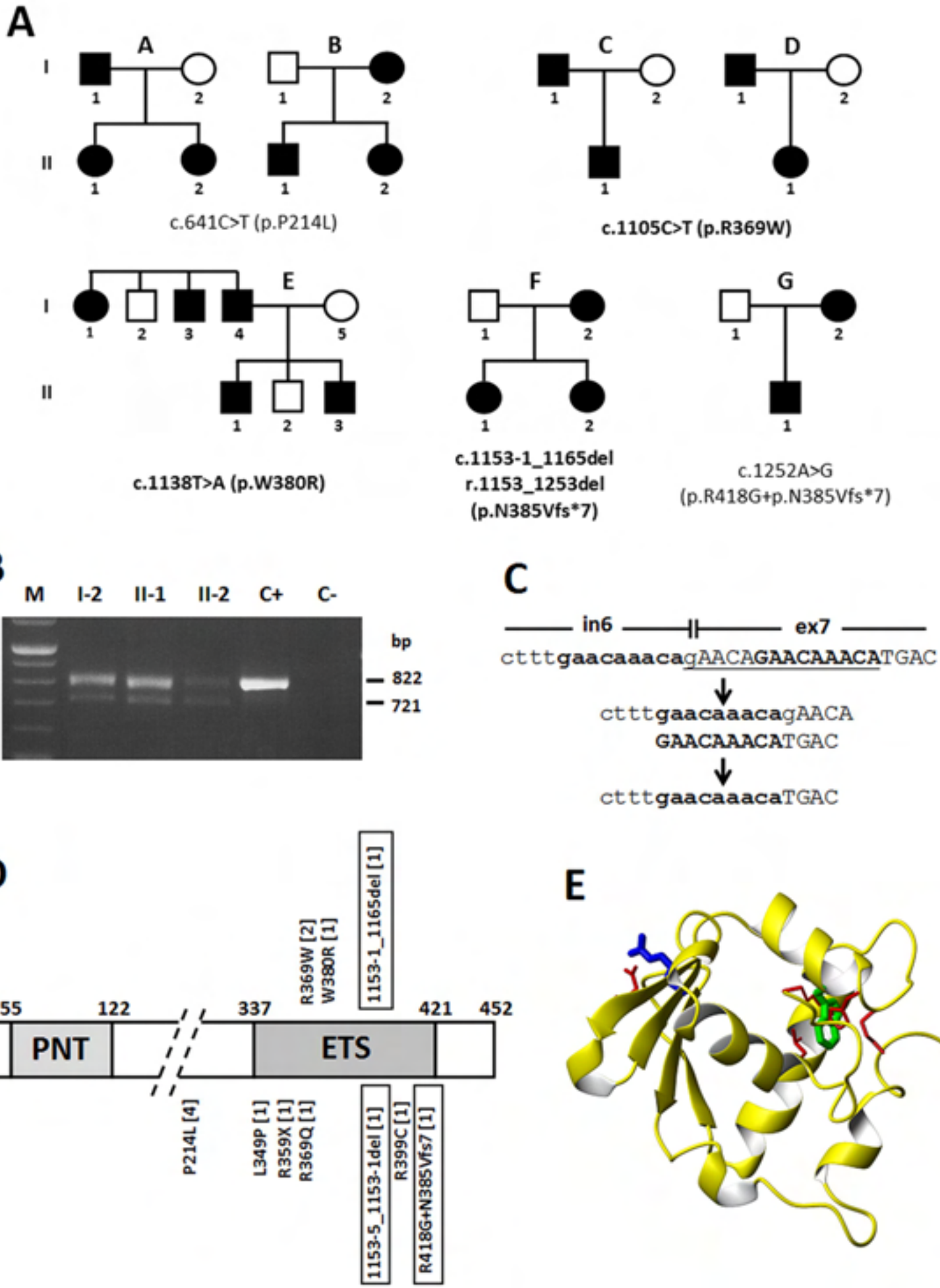
FIGURE LEGENDS

Figure 1. Mutations identified in the *ETV6* gene. (A) Pedigrees of families enrolled in this study carrying different mutations as indicated (in bold novel mutations). Nucleotide numbering reflects the ETV6 cDNA with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence (RefSeq NM_001987.4). Therefore, the initiation codon is residue 1 in the amino acid sequence. Families B and G have been previously reported (reference 4). **(B)** RT-PCR in affected members (I-2, II-1, and II-2) of family F to determine the consequence of the c.1153-1_1165del mutation on splicing. C+, wild type control; C-, negative control. The analysis shows two fragments, the wild type (822 bp) and the exon 7 skipping (721 bp) products. **(C)** The deletion of the 14 bp (gAACAGAACAAACA) of c.1153-1_1165del is likely due to non-allelic homologous recombination between the two GAACAAACA repeats located at the intron 6 and exon 7 boundary. **(D)** Domain structure of ETV6 (XP_011518909.1) based on Pfam annotation at http://www.ncbi.nlm.nih.gov/gene/2120 (PNT, N-terminal pointed domain; ETS, C-terminal DNA binding domain), with mutations identified in *ETV6*, already reported or identified in this study (top). The number of families carrying each mutation are in brackets. Mutations leading to skipping of exon 7 are boxed. **(E)** Structural modeling of the ETS domain with residues R369 (blue) and W380 (green) affected by the p.R369W and p.W380R mutations.

Figure 2. Normal differentiation but decreased ploidy by *ETV6*-RT megakaryocytes. Hematopoietic progenitors from peripheral blood samples of healthy controls (CTRL) and patients (*ETV6*-RT) were differentiated in vitro into megakaryocytes in presence of TPO, IL6 and IL11. **(A)** Representative immunofluorescence staining of plasma membrane GPIIIa in CTRL and *ETV6*-RT megakaryocytes (red=GPIIIa; blue=nuclei; scale bar=20 µm). **(B)** Flow cytometry analysis of GPIIb and GPIbα expression revealed comparable percentage of double stained population in CTRL and *ETV6*-RT at the end of the culture. **(C)** Ploidy of megakaryocytes at the end of the culture was significantly reduced in cells of *ETV6*-RT patients (*p<0.05). **(D)** Diameters of megakaryocytes were also significantly lower in *ETV6*-RT patients (total number of analyzed cells: 1,100, *p<0.01).

Figure 3. Aberrant proplatelet formation by *ETV6*-RT megakaryocytes (A) Representative light microscopy analysis of proplatelet formation and structure from controls (CTRL, i) and patients (*ETV6*-RT, ii-v) megakaryocytes cultured for 16 hours in suspension (scale bar=50 µm). **(B)** The percentage of proplatelet forming megakaryocytes was calculated as the number of megakaryocytes displaying at least one filamentous pseudopod with respect to total number of round megakaryocytes per analyzed field (*p<0.01). **(C)** Representative fluorescence microscopy analysis of proplatelet formation and structure from CTRL (i-ii) and *ETV6*-RT (iii-vi) megakaryocytes cultured for 16 hours in adhesion on fibrinogen. Pictures

clearly show defective proplatelet elongation in *ETV6*-RT (red=β1-tubulin; blue=nuclei; scale bar=30 µm). **(D)** The percentage of proplatelet forming megakaryocytes was calculated as the number of β1-tubulin+ cells displaying at least one pseudopod with respect to total number of round megakaryocytes per analyzed field (*$p<0.01$).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c.1138T>A (p.W380R)
c.1153-1_1165del
r.1153_1253del
(p.N385Vfs*7)
c.1252A>G
(p.R418G+p.N385Vfs*7)
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I-2
II-1
II-2
C+
C-
bp
822
721
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ex7
ctttgaacaaacagAACAGAACAAACATGAC
ctttgaacaaacagAACA
GAACAAACATGAC
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R369W [2]
W380R [1]
1153-1_1165del [1]
P214L [4]
L349P [1]
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R369Q [1]
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R399C [1]
R418G+N385Vfs7 [1]
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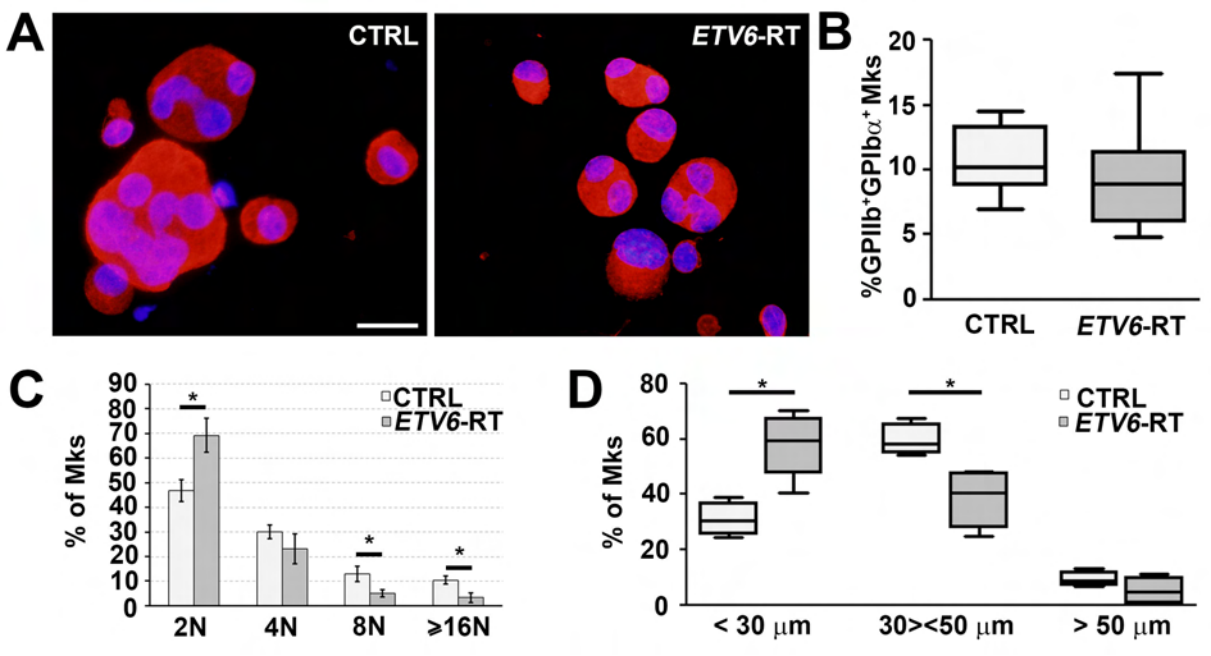
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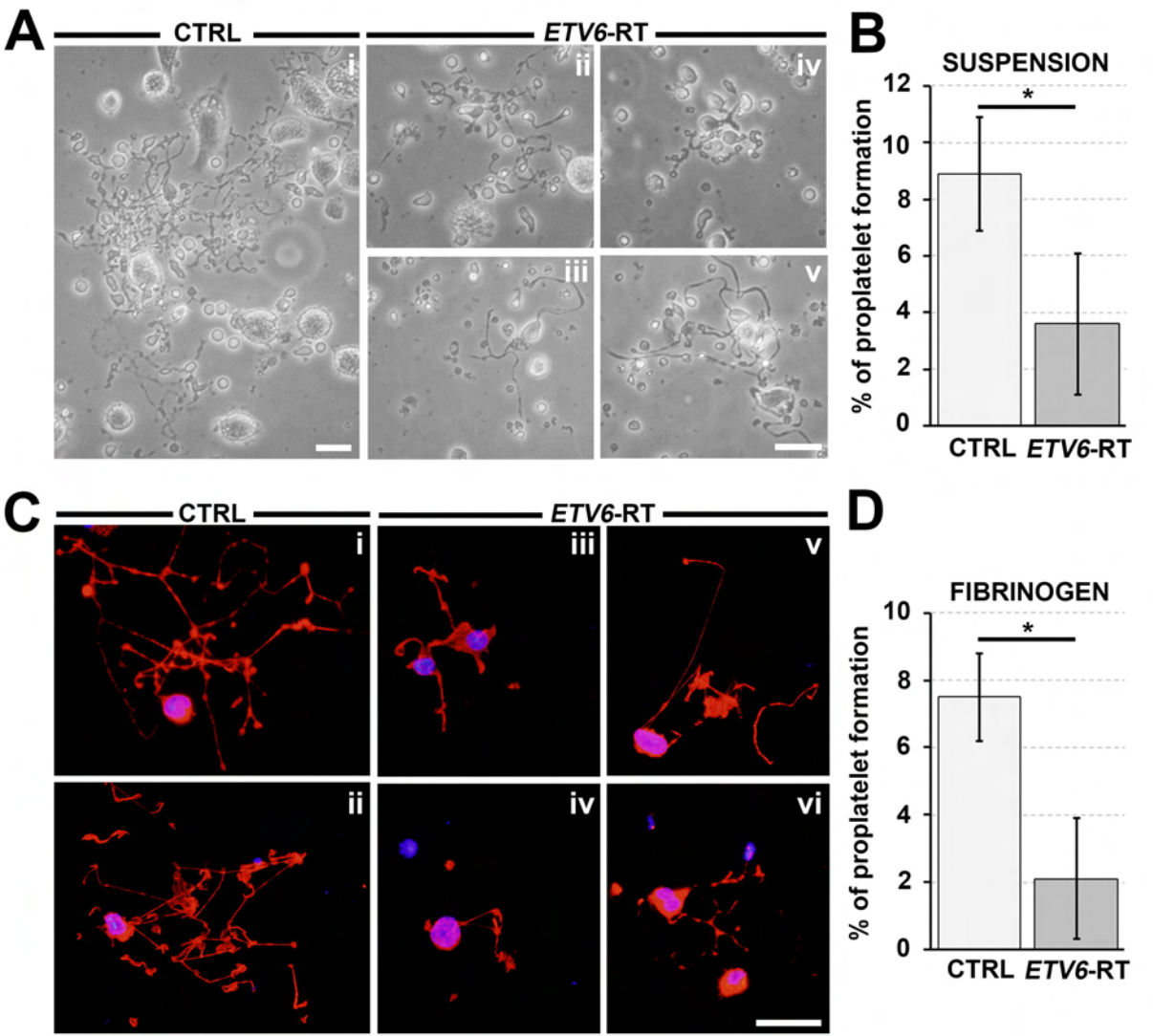
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SUPPLEMENTARY INFORMATION OF THE MANUSCRIPT ENTITLED "CLINICAL AND PATHOGENETIC FEATURES OF *ETV6*-RELATED THROMBOCYTOPENIA WITH PREDISPOSITION TO CHILDHOOD ACUTE LYMPHOBLASTIC LEUKEMIA", by Melazzini et al.

SUPPLEMENTARY METHODS

Mutation screening and RT-PCR

For Sanger sequencing PCR was carried out in 35 µl of total reaction volume with 25 ng of genomic DNA, 10 µM of each primer, and Kapa 2G Fast Hot Start ReadyMix 2X (KapaBiosystems, Cape Town, South Africa). PCR products were sequenced using the ABI PRISM BigDye v3.1 Terminator Cycle Sequencing Ready Reaction Kit and ABI PRISM 3130xl sequencer (Applied Biosystems, Foster City, CA).
Whole exome sequencing (WES) was performed on DNA samples using the solid-phase NimbleGen SeqCap EZ Exome 44Mb array (Nimblegen Inc., Madison, WI) and sequenced as 91/100 bp paired-end reads on Illumina HiSeq2000 platform (Illumina Inc., San Diego, CA). Variants passing quality filters were annotated using ANNOVAR[1] against NCBI RefGene (http://www.ncbi.nlm.nih.gov).
cDNA was synthesized with the Go Script Reverse Transcription System kit (Promega, Madison, WI, USA). Amplification reactions were performed using the following pairs of primers: F (5'-ACCAGGAGAACAACCACCAG-3') in exon 5 and R (5'-AAGTGTCCCTGCCATTTCTG-3') in exon 8 using DNA polymerase KAPA2G Fast HS Ready Mix (Kapa Biosystems, Wilmington, MA, USA). PCR products were sequenced as indicated above.

Bioinformatic tools and analysis of the structures

Missense variants were evaluated using prediction programs, such as PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/), Mutation Taster (http://www.mutationtaster.org/) Mutation Assessor (http://mutationassessor.org/), and SIFT (http://sift.jcvi.org). The only available structure of an ETV6 construct containing residues C338 to L442 (numbered in the file C8 and L112 using the construct numbering) was obtained from the PDB database. The structure was displayed by the graphic program MOLMOL and analyzed by the DSSP program to assess water exposure.

Measurement of platelet diameters

Platelet diameters were measured on May-Grünwald-Giemsa-stained blood smears by software-assisted image analysis (Axiovision 4.5, Carl Zeiss, Gottingen, Germany). On average, 200 platelets were evaluated in

each subject, and the maximum diameter of each platelet was recorded. A previous examination of a wide series of patients with IT showed that the percentage of large platelets can be reliably estimated without the support of image analysis, by visually comparing the diameters of platelets with those of erythrocytes and calculating the percentage of platelets larger than half an erythrocyte.[2] Thus, the percentage of large platelets was calculated also by this empirical method on MGG-stained blood smear by a blinded operator, as reported.[2]

Flow cytometry study of platelet surface glycoproteins

The following fluorescein isothiocyanate-conjugated (FITC) monoclonal antibodies (moAbs) from Immunotech (Marseille, France) were used: SZ21 that recognizes GPIIIa (CD61), P2 recognizing GPIIb in the intact complex with GPIIIa (CD41), SZ1 recognizing GPIX when correctly complexed with GPIbα and SZ2 against GPIbα (CD42b).

Platelet aggregation

Platelet aggregation was investigated according to the densitometric method of Born in response to the following agonists: collagen (4 and 20 µg/mL; Mascia Brunelli, Milan, Italy), adenosine diphosphate (ADP, 5 and 20mM) and ristocetin (1.5 mg/mL), both from Sigma-Aldrich (St Louis, MO).

Flow cytometry study of platelet activation

Platelet activation in response to ADP or TRAP (Tocris Bioscience, Bristol, UK) was investigated by flow cytometry.[3] Samples of patients and controls (unaffected relatives of *ETV6*-RT patients and age-matched healthy volunteers) were processed in parallel. Aliquots of whole blood were incubated with moAbs and either TRAP 25 µM, ADP 1 µM, or vehicle HEPES buffer for 10 minutes at 37°C and fixed with paraformaldehyde. The following moAbs were used: PAC1, which specifically binds to the activated conformation of GPIIb-IIIa (Becton Dickinson, San Josè, CA); CLB-Thromb/6 against P-selectin (Immunotech); SZ2 against GPIbα; P2 against GPIIb-IIIa. Platelets were gated by GPIIb-IIIa expression. Platelet activation was expressed as the ratio between mean fluorescence intensity (MFI) measured after stimulation with each agonist and MFI measured after incubation with the buffer alone.

Platelet adhesion and spreading

Glass coverslips were coated with type I collagen, von Willebrand Factor, or fibrinogen, and blocked with bovine serum albumin (BSA), as reported.[4] Washed platelets were prepared [5] and aliquots of 1 x 10^8 platelets in 2.5 mL HEPES with 1 mg/mL BSA, 5.5 mM glucose and 2 mM $MgCl_2$ were incubated on coated coverslips for 45 minutes at 37°C. After washing, specimens were fixed and stained with Alexa Fluor 488-conjugated phalloidin (Life Technologies, Carlsbad, CA) as reported.[4] Samples of patients and controls were processed simultaneously. At least 10 random microscopic fields at 63x magnification were acquired per each specimen for image analysis, which was performed by the Axiovision 4.6 software (Carl Zeiss, Oberkochen, Germany). The following parameters were assessed: number of adherent platelets, percentage of spread platelets and average platelet area.

Differentiation of human megakaryocytes and morphological analysis

$CD45^+$ cells from peripheral blood samples were separated by immunomagnetic bead selection (Miltenyi Biotech, Bologna, Italy), cultured in Stem Span medium (StemCell Technologies, Canada) and analyzed using an Olympus BX51 microscope (Olympus, Deutschland GmbH, Hamburg, Germany) as previously described.[6,7] Mk diameters were measured using the LCmicro software by Olympus Soft Imaging Solutions GmbH. Samples of patients and controls were processed in parallel.

Megakaryocyte flow cytometry

To analyze the percentage of fully differentiated Mks at the end of the culture, 200 x 10^3 cells were recovered, washed with PBS, and double-stained with the FITC-conjugated antibody HIP8 against GPIIb (eBioscience, Milan, Italy) and the PE-conjugated antibody HIP1 against GPIbα (Abcam, Cambridge, UK). Cells were analyzed using a Navios flow cytometer (Beckman Coulter). The analysis of DNA content was performed as described.[8] A minimum of 10,000 Mks were acquired. Ploidy was analyzed upon gating $GPIIb^+$ events.

Evaluation of proplatelet formation by *in vitro* differentiated megakaryocytes

Proplatelet yields were evaluated both in suspension and in adhesion on fibrinogen at the end of the culture (14th day), as previously described.[6,9] Proplatelets on fibrinogen were stained with rabbit anti-β1-tubulin primary antibody (kindly provided by Prof. J. Italiano Jr.) and Alexa Fluor-conjugated anti-rabbit antibody (Life Technologies). The size of proplatelet tips was measured by image analysis as previously described.[6]

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Supplementary Table S1. Prediction of the effect of the two missense variants identified in families C, D, and E on protein function by different tools.

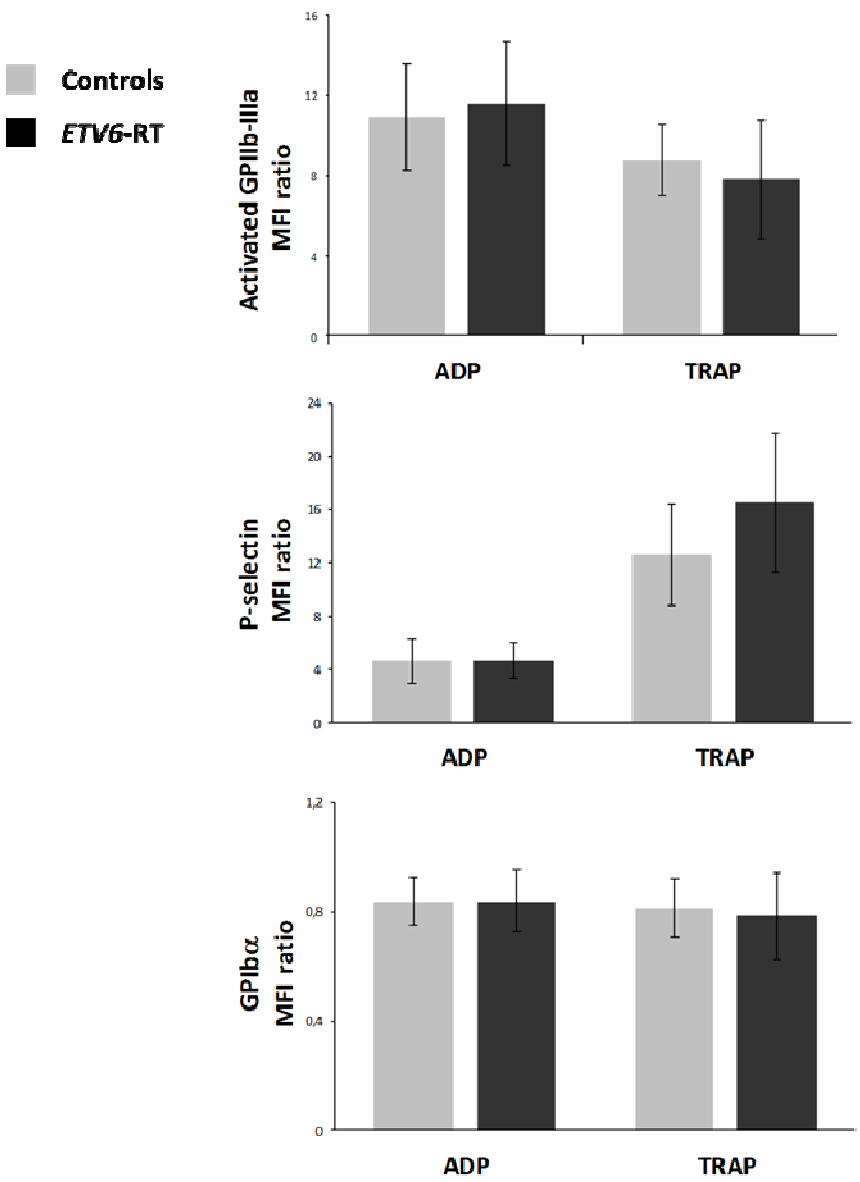
	Mutation Taster	**Mutation Assessor**	**Polyphen**
p.R369W	Disease-causing	Medium	Probably damaging
p.W380R	Disease-causing	High	Probably damaging

Supplementary Table S2. Summary of the available platelet counts in the investigated patients.

Family/ Individual	No. of measurements[1]	Age - range[2], y	Platelets, $x10^9$/L - range (median)
A/I-1	3	48-57	83-125 (110)
A/II-1	7	3-20	41-70 (61)
A/II-2 [3]	7	9-27	62-97 (84)
B/I-2 [3]	21	17-43	70-119 (94)
B/II-1	29	0-15	62-105 (71)
B/II-21	25	2-18	31-85 (55)
C/I-1	2	38-48	84-112 (98)
C/II-1	4	3-13	55-87 (79)
D/I-1	2	47-53	110-137 (123)
D/II-1	4	1-7	97-113 (103)
E/I-1	1	37	105
E/I-3[4]	3	7-32	55-71 (63)
E/I-4	3	43-45	93-109 (98)
E/II-1[5]	3	4-6	48-72 (60)
E/II-3	15	0-13	75-114 (99)
F/I-2	6	30-49	77-115 (107)
F/II-1	5	5-12	57-95 (65)
F/II-2 [3]	5	5-17	59-70 (64)
G/I-2	5	20-51	85-110 (95)
G/II-1	5	3-28	80-105 (99)

Notes: [1] Number of the available measurements of automated platelet count (including last evaluation). [2] Range of the age when the available measurements have been performed. [3] Platelet counts measured during chemotherapy for ALL were not included. [4] Platelet counts measured during chemotherapy for ALL and after HSCT were not included. 5 Platelet count measured after the diagnosis of polycythemia vera were not included.

SUPPLEMENTARY FIGURE 1



Supplementary Figure 1. Flow cytometry analysis of platelet activation in response to ADP or TRAP in *ETV6*-RT patients. The expression of activated GPIIb-IIIa (GPIIb-IIIa, PAC1 antibody binding), P selectin and GPIbα was measured after the addition of TRAP 25 µM, ADP 1 µM or the vehicle buffer alone. Data are expressed as the ratio between mean fluorescence intensity (MFI) measured after stimulation with each agonist and MFI measured after incubation with buffer alone. No significant differences were observed between 11 *ETV6*-RT patients and 20 healthy subjects analyzed in parallel (two tailed Student's t test).